Supplementary Information (SI) for Journal of Materials Chemistry B.
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Supplementary material

Co-Assembled Ion-pair Complex Nanoparticles from Tranexamic Acid and Metformin with Enhanced Transdermal Efficacy against UVB-Induced Pigmentation

Supplementary Methods

S1 Preparation of Rho B-PMT and Rho B-S-PMT

The preparation of Rho B-PMT and Rho B-S-PMT followed a procedure analogous method for PMT or S-PMT, purified water was replaced with a Rhodamine B (Rho B) solution (0.5 mg/mL) or Rho B solution (0.5 mg/mL) with an additional SNAC (25 mg/mL). This Rho B solution was prepared by dissolving 5 mg of Rhodamine B in 10 mL of purified water.

S2 ^1H NMR

^1H NMR spectra including tranexamic acid (TXA), metformin (Met), pEGCG, and PMT were recorded using a Bruker Avance NEO 600 spectrometer at a frequency of 600 MHz. The measurements were performed at 30 °C using deuterium oxide (D_2O) as the solvent. Acquisition parameters included 4 dummy scans, 8 scans, a spectral window of 20.02 ppm, an acquisition time of 3.27 s, and a relaxation delay (D1) of 14.091 s, ensuring a signal-to-noise ratio > 500. For sample preparation, approximately 20 mg of each compound was dissolved in 0.55 mL of D_2O, and the resulting solution was transferred into a 5 mm NMR tube. Data processing was carried out using MestreNova v.14.2.2 software (Mestrelab Research, Spain).

S3 TEM sample

The copper grids were immersed in appropriately diluted sample suspensions for 10 min. The grid for TEM was then retrieved, blotted onto filter paper, and air-dried at room temperature; meanwhile, the grid was dried in a vacuum oven at 35 °C. Subsequently, a 2% (w/v) phosphotungstic acid solution was applied to the TEM grid for negative staining.

S4 Determination of TXA Content by HPLC

The HPLC analysis was performed on a Prominence CBM-20A/20Lite system (Shimadzu, Japan) equipped with a C18 column (5 μm, 4.6 × 250 mm; Dalian Yilite Analytical Instrument Co., Ltd). The mobile phase for tranexamic acid detection consisted of 0.23% sodium dodecyl sulfate solution and methanol (45:55, v/v). The 0.23% sodium dodecyl sulfate (SDS) solution was prepared by dissolving 18.3 g of anhydrous sodium dihydrogen phosphate in 800 mL of purified water, followed by the addition of 8.3 mL triethylamine and 2.3 g SDS; the resulting solution was then adjusted to pH 2.5 with phosphoric acid and finally diluted to 1000 mL with purified water. Chromatographic separation was conducted under the following conditions: column temperature 30 °C, flow rate 0.5 mL/min, injection volume 20

μL, and UV detection at 220 nm.

S5 Determination of Met Content by ultraviolet-visible spectrophotometry (UV-Vis)

A UV-1800 spectrophotometer (Shimadzu, Japan) was employed for the analysis. Met was diluted in PBS and its maximum absorption wavelength was identified at 233 nm by full-wavelength scanning. A standard curve was established by measuring the UV absorbance of Met solutions at varying concentrations, with Met concentration plotted on the x-axis and absorbance on the y-axis. Linear regression yielded the equation y = 0.0756x + 0.02804 (R^2 = 0.999), indicating good linearity between Met concentration and absorbance in the range of 1-25 μg/mL. For quantification, Met-containing samples were diluted into this linear range and analyzed by UV spectrophotometry.

S6 pH Stability Study

Citrate-phosphate buffer (50 mM) was prepared by dissolving 302 mg of hydrogen phosphate dihydrate and 160 mg of citric acid in 50 mL of purified water. Subsequently, 4 parts of 5 mL of the buffer were taken and adjusted their pH to 5, 6, 7, and 8, respectively. Then, 0.5 mL of the S-PMT solution was mixed with different pH citrate-phosphate buffer and stored at room temperature for 2 h. Particle size, polydispersity index (PDI), and zeta potential were measured before mixing and after 2 h incubation at different pH condition.

S7 The cumulative permeation amount per unit area and Steady-state transdermal rate per unit area

The cumulative permeability per unit area was calculated as follow:

$$Q = (V \times C_n + \sum_{i=1}^{i=n-1} C_i)/A$$

In the above formula: Q is the cumulative drug penetration per unit of skin area，V was the volume of the receiving cell, C_n was the drug concentration measured by sampling at the nth point, and A was the effective permeation area of the diffusion cell. The steady-state transdermal rate per unit area was the slope of the cumulative drug penetration per unit area versus the linear part of the time plot.

S8 Haemolysis testing

Red blood cells (RBCs) were obtained by centrifuging whole blood at 1500 rpm for 15 min and discarding the supernatant. The erythrocytes were diluted with saline solution containing S-PMT nanoparticles solution (0, 10, 50, 100, 200, 300, 400 and 500 μg/mL). Saline and deionized water diluted red blood cells (RBCs) as the negative control and positive control, respectively. The mixtures were incubated at 37 °C for 4 h and then centrifuged at 3000 rpm for 10 min. The obtained supernatants were collected, the absorption value at 540 nm were measured with a UV-vis spectrophotometer. Hemolysis rate followed as the formula:

$$\text{Hemolysis rate (\%)} = \frac{(A_x - A_-)}{(A_+ - A_-)} \times 100\%.$$

A_x, A_+, and A_- represented the absorbance at 540 nm of S-PMT nanoparticles solution, H_2O, and Saline.

S9 Melanin standard curve

Melanin standard was weighed and prepared as a 2 mg /mL melanin standard solution using 1 mol/ L NaOH solution. The standard solution was subjected to gradient dilution to obtain melanin solutions of different concentrations (20, 40, 60, 80, 100 μg /mL). Absorbance values were measured at A405 using an enzyme meter to plot the standard curve.

S10 Photodamaged cell model

The L929 cell line was subjected to 50 mJ/cm^2 of UVB irradiation to establish a photodamage cell model. The cells in PBS were exposed to a narrow-spectrum UVB lamp (Philips, 311 nm) at an irradiance of 100 μW/cm^2 for 200 s. Then, the light-damaged L929 cells were incubated for 24 h in DMEM containing various formulations to evaluate their antioxidant efficacy.

S11 Transdermal Water Loss Experiment on C57BL/C Mice

Transepidermal water loss (TEWL) of mouse dorsal skin was measured using a Tewameter TM300 probe (Courage Khazaka, Germany). Except the blank control group, C57BL/C mice model of UVB-induced skin pigmentation was established following the protocal in Section 2.9. In addition to the blank control group, the mice in the UVB, TXA, Met, T/M, and S-PMT groups (n=3) received UVB irradiation at a dose of 180 mJ/cm² on the dorsal skin while using the different formulations. TEWL values (g/m² h) of the dorsal skin of each mouse on day 14 were recorded using a Tewameter TM300.

S12 H&E staining

The tissue sections were baked in an oven at 65 °C for 1 h, and then deparaffinised with xylene for 15 min. After deparaffinisation, the tissue sections were stained with hematoxylin for 5 min and then rinsed with PBS. Cells were differentiated with 1% hydrochloric acid alcohol and rinsed with running water for several min. Stain with 0.1-1% lithium carbonate counterblue for 5 min, followed by rinsing; cells were stained with poncoronate violet stain for 5 min, followed by rinsing. and the HE staining sections were visualized using a light microscope (BZ-X810).

S13 Fontana-Masson (FM staining) and Masson's staining

Deparaffinized and rehydrated tissue sections were subjected to FM staining. The ammoniacal silver solution was freshly prepared by titrating 5% silver nitrate with concentrated ammonia water until the initial precipitate dissolved, followed by the addition of a few drops of 5% silver nitrate to achieve a slight opalescence. Sections were impregnated with this solution and incubated in the dark at room temperature for 12 h. After thorough washing, sections were counterstained with Verhoeff's Van Gieson (VG) solution—a mixture of 1% acid fuchsin and saturated aqueous picric acid in a 1:9 (v/v) ratio for 5 min. Finally, the sections were dehydrated through a graded ethanol series, cleared in xylene, and mounted with neutral balsam.

After deparaffinisation, tissue sections were stained with Masson's stain in 1% phosphomolybdic

acid solution for 5 min and 2% glacial acetic acid solution for 1 min; cells were counterstained with aniline blue solution for 5 min and rinsed in 2% glacial acetic acid solution for 1 min. The cells were dehydrated with anhydrous ethanol 3 times for 5 seconds each time, permeabilised with xylene 3 times for 1 min each time, and sealed with neutral gum and coverslips. The cells were observed under a light microscope and photographed at 200× magnification. The nuclei were blue-black, the cytoplasm was red, and the collagen fibres were blue.

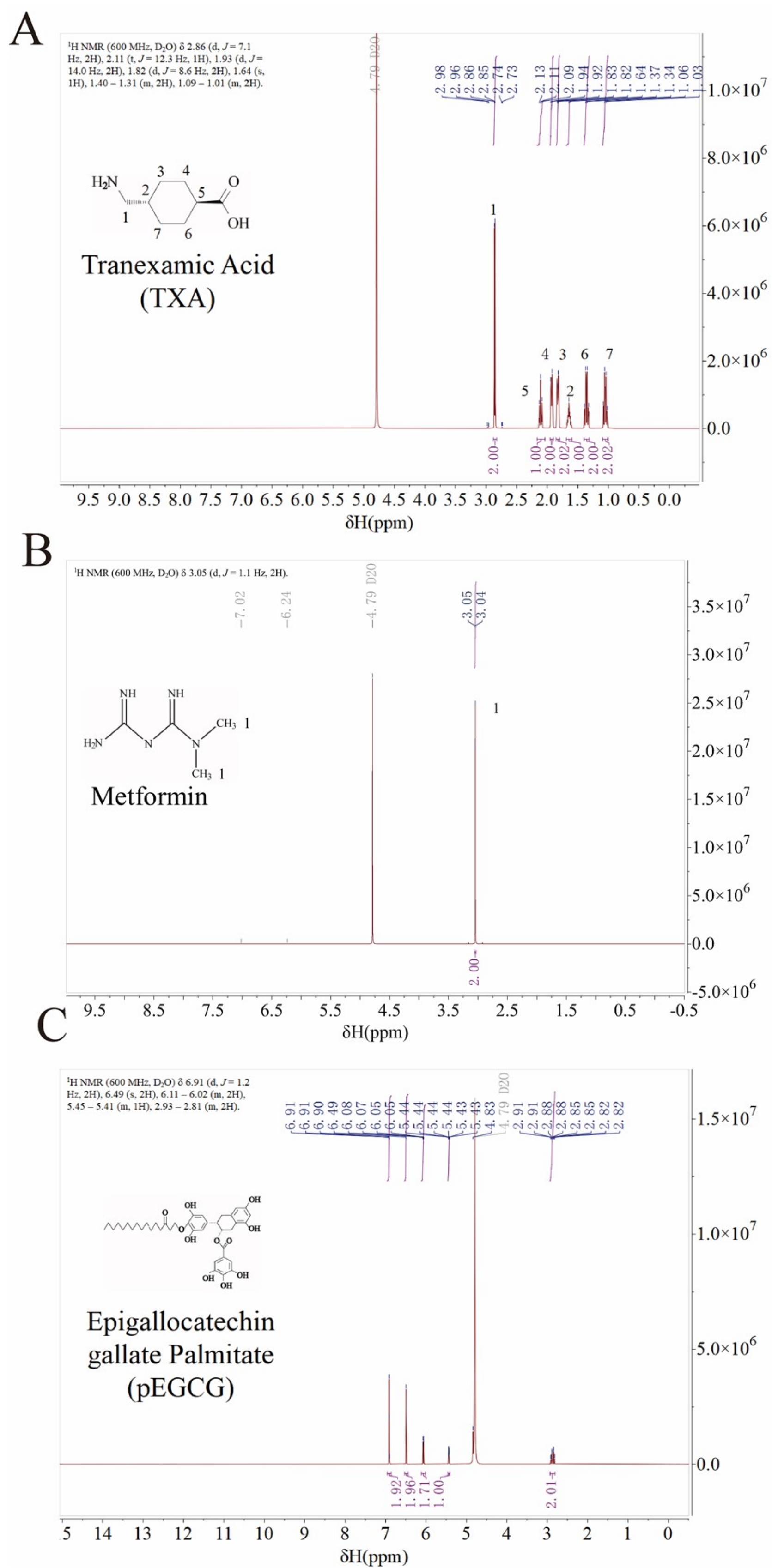
S14 Western Blot Analysis

Protein extraction from skin tissue Skin tissue samples were retrieved from –80 °C, quickly weighed (approximately 90 mg) and excised after removing hair. A fresh lysis buffer was prepared on ice by mixing RIPA lysis buffer with protease inhibitor (50×) and phosphatase inhibitor (50×) at a ratio of 50:1:1 (e.g., 1 mL RIPA containing 20 μL each of protease and phosphatase inhibitors). The tissue pieces were added to the pre-cooled lysis buffer (1000 μL for 90 mg tissue) and thoroughly homogenized using a handheld grinder on ice until no visible clumps remained. The homogenate was incubated on ice for 25 min with vortexing every 5 min, then centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was carefully transferred to a new tube without disturbing the pellet to determine protein concentation or stored at –80 °C. Protein concentration was determined by the BCA kit after a 10-fold dilution (standard curve range 0–0.5 μg/μL). Samples were then adjusted to the concentration (approximately 2 mg/mL), mixed with 5× loading buffer (4:1 ratio), and denatured at 100 °C for 10 min.

Western Blot Analysis For SDS-PAGE, protein samples of skin tissue (20–30 μg per lane) were separated on 12% polyacrylamide gels at 80 V for 30 min followed by 120 V for 1.5 h. The resolved proteins were transferred onto a PVDF membrane (activated with methanol) at 400 mA for 60 min in an ice-cooled transfer system. The membrane was blocked with 5% BSA in TBST for phosphoproteins at room temperature for 1 h, then incubated overnight at 4 °C with primary antibodies (diluted in blocking buffer according to the manufacturers' instructions) targeting the following proteins: phospho-p38 rabbit mAb, phospho-PKA rabbit pAb, MITF polyclonal antibody, tyrosinase rabbit pAb, and GAPDH rabbit polyclonal. After washing three times with TBST (10 min each), the membrane was incubated with HRP-conjugated secondary antibody (diluted in blocking buffer) for 1.5 h at room temperature, followed by three TBST washes. Immunoreactive bands were visualized using an ECL substrate and a chemiluminescence imaging system. Membranes were subsequently stripped and reprobed with an internal control antibody (GAPDH) following the same procedure.

S15 Irritation experiment of S-PMT on C57BL/C mice

Nine naive mice were selected for the Irritation study. Twenty-four hours prior to experiment initiation, the dorsal skin of each mouse was carefully inspected to confirm the absence of pre-existing lesions. Following anesthesia, the dorsal hair was shaved to create a 2 × 3 cm^2 area while ensuring no skin damage occurred during the procedure. Sterile 2 × 2 cm^2 gauze squares were prepared by autoclaving for subsequent application. The positive control group received 10% formaldehyde solution, the negative control group was treated with normal saline, and the experimental group was administered S-PMT containing 20 mg/mL TXA. Each test solution (1 mL) was applied to sterilized gauze, which was then placed on the prepared dorsal skin and secured with medical tape. Skin reactions were evaluated at 8, 16, and 24 h intervals, with erythema and edema severity scored according to established criteria. The mean scores from all three groups were calculated for comprehensive assessment, with detailed scoring criteria provided in Tables S2 and S3.



Supplementary Figures

Fig. S1 ^{1}H NMR spectra of TXA (A), Met (B) and pEGCG (C).

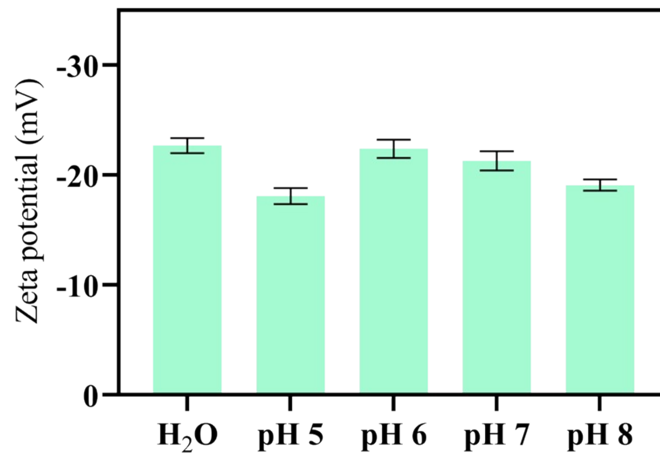


Fig. S2 Zeta potential of S-PMT at different pH condition.

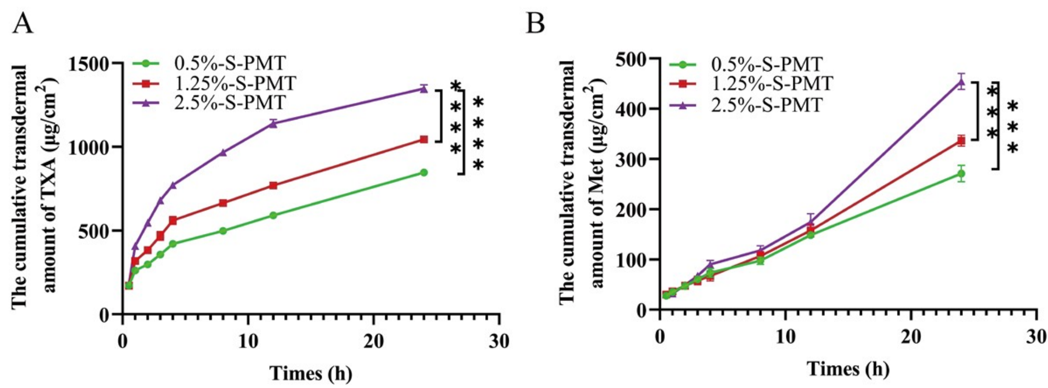


Fig. S3. *In vitro* cumulative permeation of TXA（A）and Met (B) per unit area from PMT solutions containing different concentrations of SNAC (mean ± SD, n = 3,****P* < 0.001, *****P* < 0.0001).

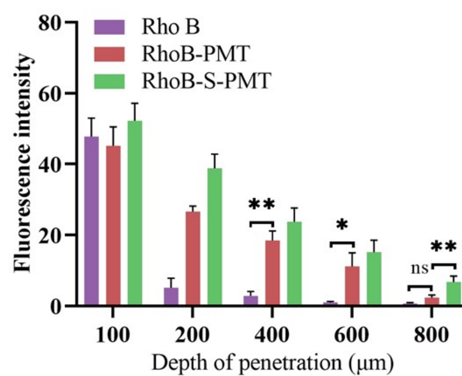


Fig. S4. Quantification of fluorescence levels of Rho B preparations at different penetration depths using ImageJ software (mean ± SD, n = 3, ns no significant difference, **P* < 0.05, ***P* < 0.01).

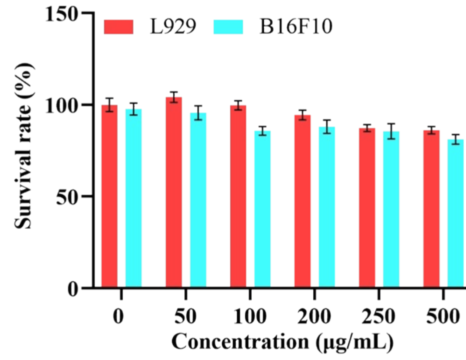


Fig. S5. Biocompatibility of S-PMT on L929 and B16F10 cells

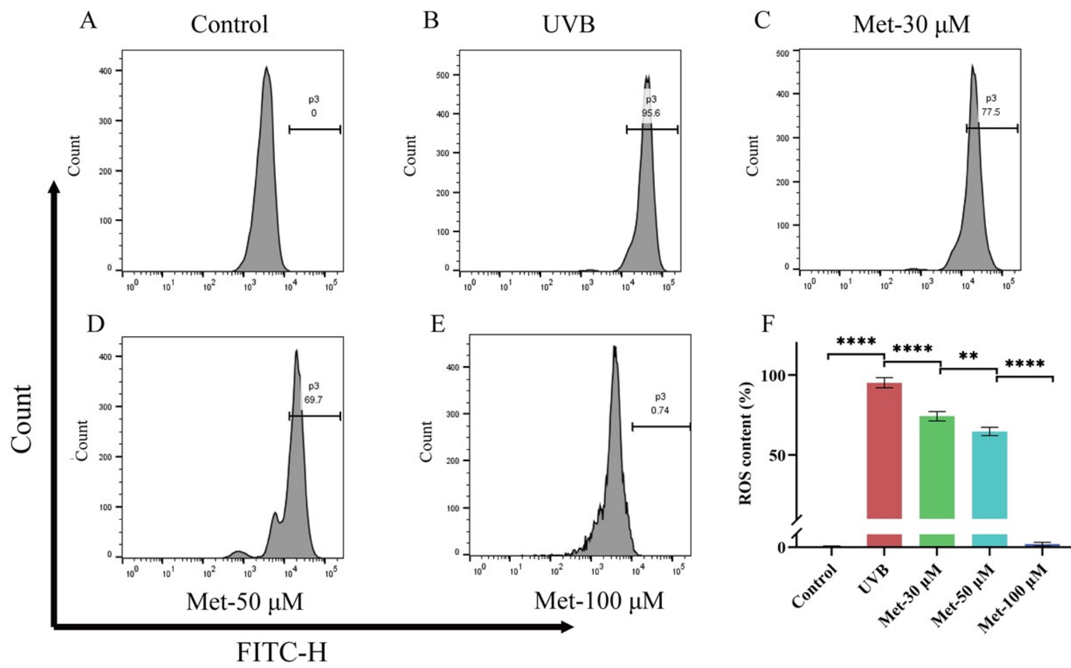


Fig. S6. Suppressive effects of Met on UVB-induced oxidative stress in L929 cells using flow cytometry, (A)-(E) The control group, UVB model group, and Met treatment groups with different concentrations (30 μM, 50 μM,100 μM, (F) ROS content in different groups (mean ± SD, n = 3,**P < 0.01, ***P < 0.001, ****P < 0.0001).

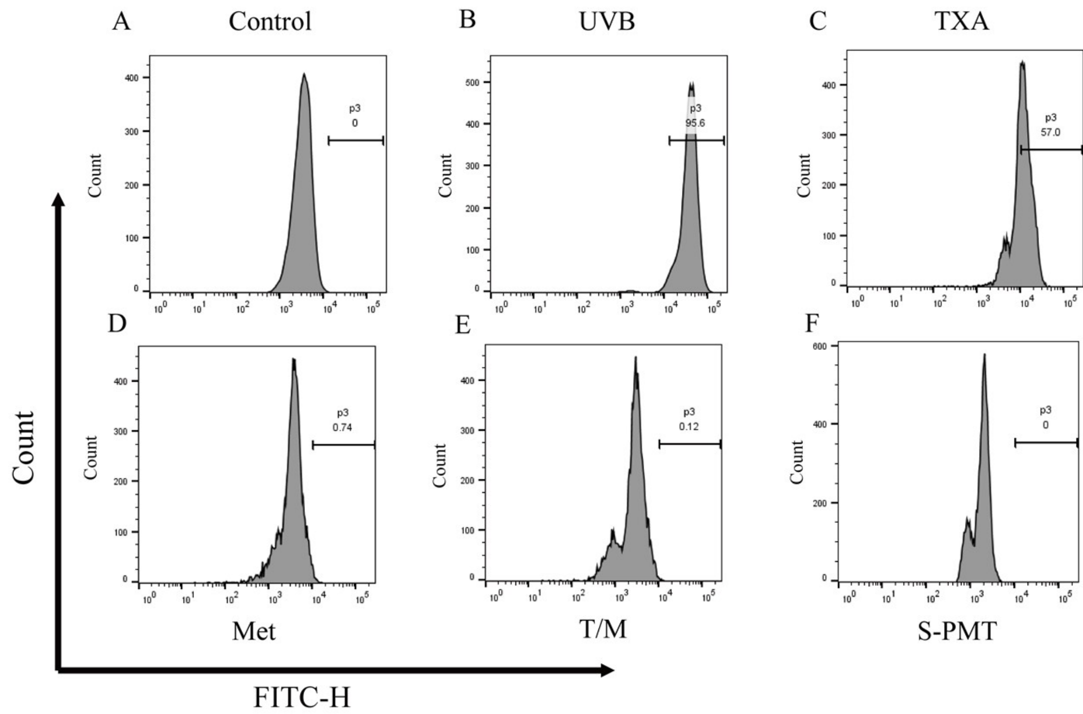


Fig. S7. Inhibitory effects of different formulations on UVB-induced oxidative stress in L929 cells using flow cytometry

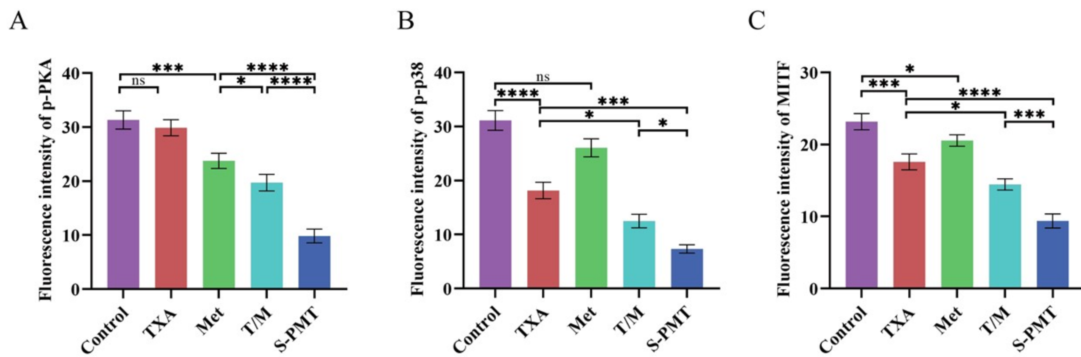


Fig. S8. Quantitative analysis of the fluorescence intensity of p-PKA (A), p-p38 (B) and MITF (C) in B16F10 using ImageJ software (mean ± SD, n = 3,ns no significant difference, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$）

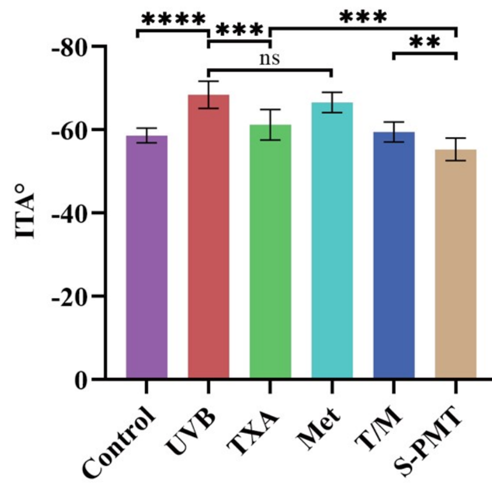


Fig. S9. Quantitative analysis of ITA° in mouse ear (mean ± SD, n = 3, ns no significant difference, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001)

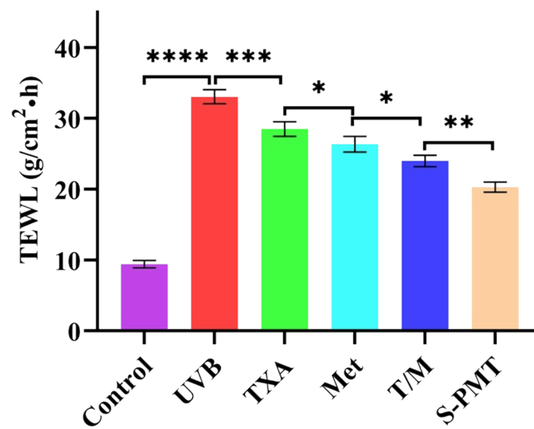


Fig. S10. Transdermal dehydration of mouse skin (mean ± SD, n = 3, ns no significant difference, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001)

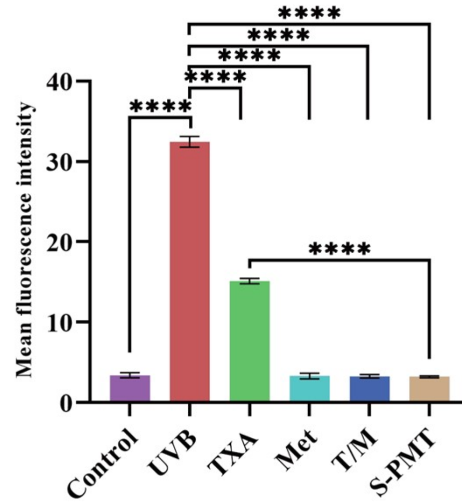


Fig. S11 Quantitative analysis of the fluorescence intensity of DHE in mouse skin using ImageJ software (mean ± SD, n = 3, ns no significant difference, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001)

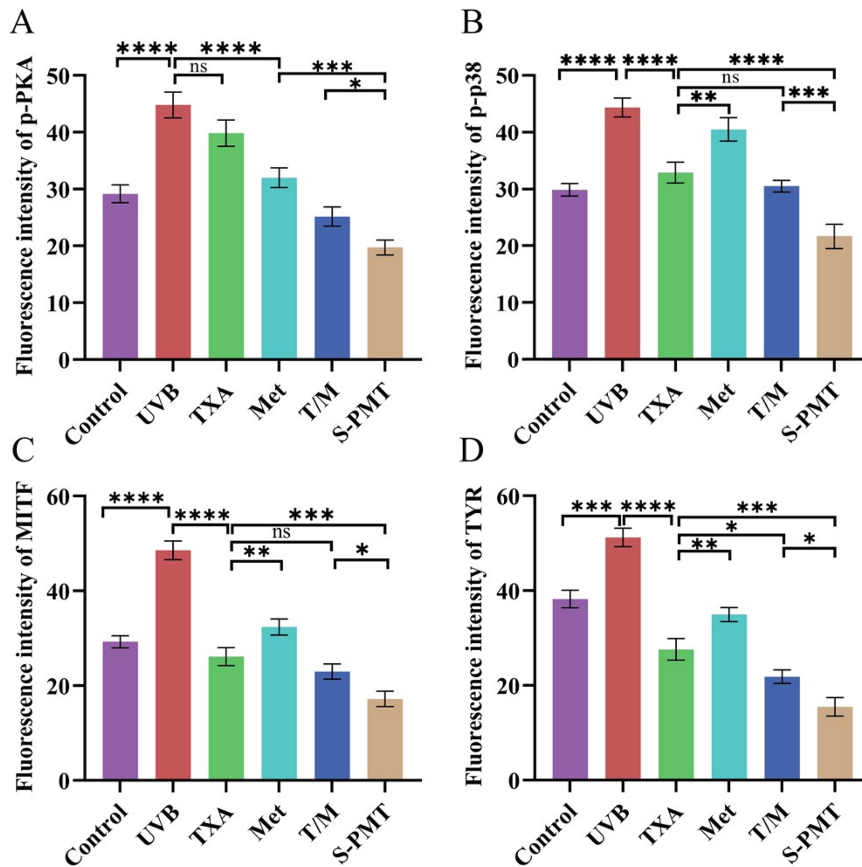
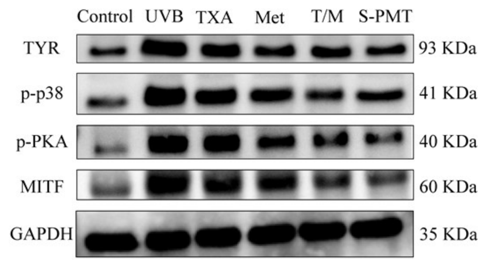


Fig. S12. Quantitative analysis of the fluorescence intensity of p-PKA (A), p-p38 (B), MITF (C), and TYR (D) in mouse Skin using ImageJ software (mean ± SD, n = 3,ns no significant difference, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).



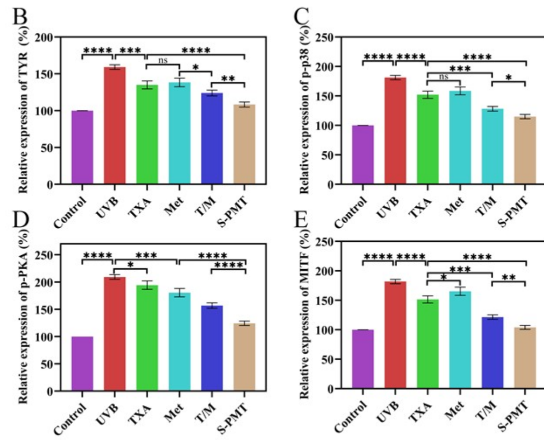


Fig. S13 Western blot analysis of TYR, p-p38, p-PKA, MITF, and GAPDH proteins in mouse dorsal skin (A). Quantitative analysis of the relative expression levels of TYR (B), p-p38 (C), p-PKA (D), and MITF (E) proteins in mouse dorsal skin by western blot analysis. Each value was calculated as the ratio of signal intensity compared to that of GAPDH. (mean ± SD, n = 3; ns, no significant difference; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$).

Supplementary Tablets

Tab. S1 Antibodies and Kits used in the work

Antibodies and Kits	Company
Phospho-p38 Rabbit mAb	ABclonal
Phospho-PKA Rabbit pAb	Bioss
MITF Polyclonal antibody	Proteintech
Tyrosinase Rabbit pAb	Bioss
Goat Anti-rabbit IgG/Alexa Flour 594	Bioss
Sodium Citrate Antigen Retrieval Solution	Beijing Applygen Technologies Inc.
TRIS-EDTA Antigen Retrieval Solution	Biosharp
Alexa Fluor 594-conjugated anti-rabbit antibody	Wuhan Kerui Biotechnology Co., Ltd.
GAPDH Polyclonal antibody	Proteintech
HRP-conjugated Goat Anti-Rabbit IgG(H+L)	Proteintech
Tyrosinase activity assay kit	Beijing Solarbio Science & Technology Co.Ltd.
Lipid oxidation (MDA) test kit	Shanghai Beyotime Biotechnology Co., Ltd
Mouse IL-1β Precoated ELISA kit	Dakewe Biotech Co., Ltd.
Mouse IL-6 Precoated ELISA kit	Dakewe Biotech Co., Ltd.
Mounting Medium with DAPI Aqueous	Abcam

Tab. S2 Skin irritation judging criteria

Skin condition		score
Erythema and scab formation	No erythema seen	0
	Slight erythema (small dots)）	1
	Visible erythema (small dots)	2
	Severe erythema (rash in red patches)	3
	Severe erythema with crust formation	4
edema formation	No edema seen	0
	slight edema	1
	Slightly edematous Boundaries of the bulge are clear.	2
	Moderate edema raised about 1 mm	3
	Severe edema with a bulge of more than 1 mm.	4

Tab. S3 Compound skin irritation determination score

point average	intensity rating
0.0～	non-irritating
0.5～	mildly irritating
2.0～	moderately irritating
6.0～	highly irritating

Tab. S4 Sensitisation reaction test.

Stimulation time	Negative group	Positive group	Treated group	Integral mean value	Strength grading
0 h	0	0	0	0～0.5	No irritation
8 h	0	0.5	0	0.5～2.0	Mild irritation
24 h	0	1.75	0	2.0～6.0	Moderate irritation